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Effect of aprotinin on neutrophil function after major vascular surgery

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High-dose aprotinin reduces blood loss and blood transfusion requirements during liver transplantation and cardiac and vascular surgery. The mechanism of the haemostatic effect of aprotinin is unclear. A general effect on the anti-inflammatory response may be involved. Because leucocyte activation is part of this process, white cell function was measured in patients undergoing aortic surgery who received high-dose aprotinin therapy (n = *10*) *and was compared with the results from controls who did not* (n = *10*). *The test group received an intravenous bolus* (2×10^6 *kallikrein inhibitor units*) *of aprotinin after induction of anaesthesia followed by continuous infusion* ($0{\cdot}5 \times 10^6$ *kallikrein inhibitor units/h*) *until the end of the operation. Blood samples were obtained before operation, immediately after surgery, and 1 and 7 days after operation. Aprotinin maintained significantly better postoperative white cell function as measured by bipolar shape formation* ($P < 0{\cdot}001$), *unstimulated nitroblue tetrazolium* (*NBT*) *reduction* ($P < 0{\cdot}001$) *and chemotaxis* ($P < 0{\cdot}001$). *Endotoxin-stimulated NBT reduction was similar in both groups, indicating that neutrophils from treated individuals retained the capacity to respond to oxidative stimuli. Aortic surgery activates neutrophils* in vivo, *as reflected by impaired chemotaxis and increased superoxide production. Aprotinin protects the cells against this potentially deleterious effect without affecting their ability to respond when provoked. Whether this affects leucocyte interaction with coagulation pathways and contributes to the reduction in blood loss remains to be determined.*

Aprotinin is a low molecular weight, broad-spectrum protease inhibitor which has been used for the treatment of acute pancreatitis and conditions associated with hyperplasminaemia[1]. More recently, high-dose aprotinin has been shown to reduce operative bleeding in cardiovascular[2,3], liver transplantation[4,5] and vascular[6] surgery and may be effective in other bleeding disorders, including thrombocytopenia[7]. Earlier studies on antiproteases suggested that they may be of value in haemorrhagic shock and that patients suffering from polytraumatic shock may benefit from aprotinin treatment[8,9]. The action of aprotinin under these circumstances is difficult to ascribe to any specific antiprotease mechanism. Plasmin inhibition, kallikrein inhibition and preservation of platelet number and function, possibly by protecting against plasmin-mediated degradation of platelet membrane adhesive glycoproteins, may be important[10–15]. The drug may have effects on peripheral blood leucocytes[16], which are probably involved in the mediation of some shock-related syndromes such as the adult respiratory distress syndrome. The initial aim of using high-dose aprotinin in cardiac surgery was to ameliorate the damaging effects of cardiopulmonary bypass on the lung[13]. Previous studies have reported that neutrophil function may be impaired after surgery, but there are no reports of the effect of aprotinin in this regard. This study, therefore, measured neutrophil function in patients undergoing major vascular surgery under cover of high-dose aprotinin and compared the results with those from controls not given aprotinin.

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Patients and methods

Patients

Twenty patients were included in the study. Ten consecutive patients undergoing aortic reconstruction for occlusive disease received aprotinin and were compared with ten controls undergoing various major reconstructive surgical procedures (*Table 1*). All patients had widespread atherosclerosis confirmed by non-invasive investigations including duplex ultrasonography.

Ethical approval was granted for the trial and all patients gave written consent. The aprotinin group were initially given a test dose of 50000 kallikrein inhibitor (KI) units (Trasylol; Bayer UK, Newbury, UK) slowly over 5 min. This was followed by a bolus dose of 2×10^6 KI units at the beginning of surgery and continuous intravenous infusion of $0{\cdot}5 \times 10^6$ KI units/h until the operation was completed. Both groups were otherwise treated in an identical fashion. Heparinization was reversed by protamine sulphate at the completion of the arterial reconstruction. There were no major differences in the methods of anaesthesia between the two groups and all patients received antibiotic prophylaxis with ampicillin and flucloxacillin.

Neutrophil function assays

Venous blood was collected into heparin (10 units/ml) before operation after induction of anaesthesia, immediately after surgery, and 24 h and 1 week after operation. Total white blood cell count and differentials were performed at each time and three tests of neutrophil function undertaken within 2 h of sampling. The study was not randomized, but laboratory staff were unaware whether samples were from treated or control patients.

Bipolar shape formation assay. A volume of 0·1 ml *N*-formylmethionyleucinylphenylalanine (FMLP, 10^{-8} mol/l; Sigma, Poole, UK) was added to 0·9 ml heparinized whole blood, gently mixed and incubated at 37°C for 20 min. After brief remixing, two blood films

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Table 1 *Patient details*

Patient no.	Age (years)	Sex	Weight (kg)
Aprotinin group: aortobifemoral bypass			
1	74	F	51
2	72	F	74
3	62	F	63
4	62	F	80
5	60	M	73
6	65	F	69
7	62	M	70
8	66	M	63
9	81	M	75
10	70	M	84
Median	65·5 (62–73)		71·5 (63–77)
Controls			
Aortobifemoral bypass			
1	80	M	76
2	59	F	76
3	67	F	44
4	69	F	71
Carotid endarterectomy			
5	69	M	72
6	65	M	80
7	58	M	62
Aortic aneurysm repair			
8	64	M	71
9	70	M	88
Femorodistal bypass			
10	66	F	68
Median	66·5 (62–69)		71·5 (66–77)

Values in parentheses are 95 per cent confidence intervals

were made, fixed, stained with May–Grünwald–Giemsa stain and examined microscopically. Fifty neutrophils from each film were classified according to shape, as previously described[17]. The percentage of bipolar neutrophils was recorded as the mean of the two films. The coefficient of variation (c.v.) for this technique was 9·6 per cent.

Chemotaxis. Chemotaxis was assessed using a modified raft method[18]. Leucocyte suspensions containing more than 90 per cent neutrophils were carefully prepared by dextran sedimentation, avoiding procedures likely to activate polymorphonuclear leucocytes[19]. The cells were suspended in Hank's balanced salt solution (HBSS), pH 7·4, at a concentration of 5×10^9/litre. Preparations were judged to be greater than 95 per cent viable by trypan blue exclusion[20]. Vessels containing the leucocyte suspensions were placed on top of 3·0-μm pore filters (Millipore, Harrow, UK), which in turn were placed on absorbent discs soaked in chemoattractant (10^{-9} mol/l FMLP or 20 per cent pooled human AB serum). To assess non-directed migration, HBSS was used in place of a specific chemoattractant. In this system, the cells migrate towards the chemotaxin through the pores of the filters, which are subsequently fixed and stained. Chemotaxis was expressed as the leading front (the furthest distance two cells have migrated through the membrane). The c.v. for this technique[17] is 9·2 per cent.

Nitroblue tetrazolium reduction assay. A fresh saturated solution of nitroblue tetrazolium salt (NBT, Sigma) was prepared in 1 ml phosphate-buffered saline (PBS), pH 6·8. A volume of 0·1 ml non-viable bacterial extract (Sigma, stimulated NBT test) or 0·1 ml PBS (non-stimulated NBT test) was incubated with 0·5 ml heparinized whole blood at 37°C for 20 min. NBT solution (0·1 ml) was then added to an equal volume of each preparation and incubated at 37°C for a further 20 min. Cytospin slides were made and stained, without fixing, with methylene green. The percentage of positive (formazan-containing) cells was recorded. The c.v. for this technique is 8·7 per cent.

Statistical analysis

Statistical analysis was performed using the STATGRAPHICS statistical software package (STSC, Rockville, Maryland, USA). Differences between medians were assessed by Mann–Whitney *U* test. The calculation of 95 per cent confidence intervals used MINITAB (CleCom, Edgbaston, UK).

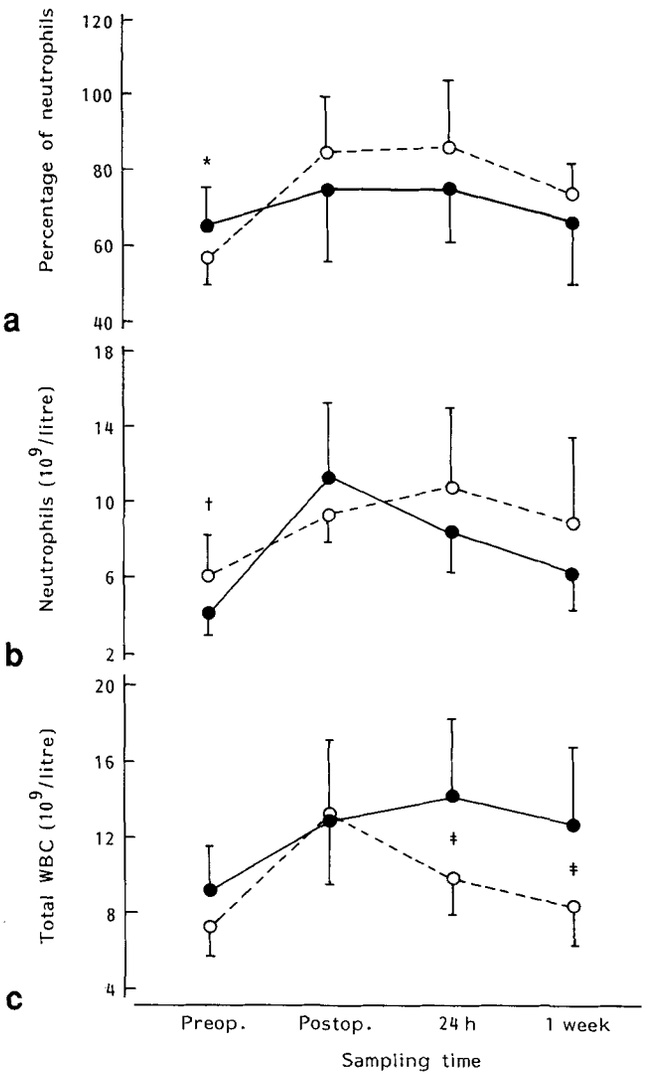

Figure 1 *Changes in* **a** *percentage neutrophils,* **b** *total neutrophil count and* **c** *total white blood cell (WBC) count in patients undergoing major vascular surgery with (●) or without (○) aprotinin. Values are median (95 per cent confidence interval).* *P < 0·01; †P < 0·02; ‡P < 0·005 *(Mann–Whitney* U *test)*

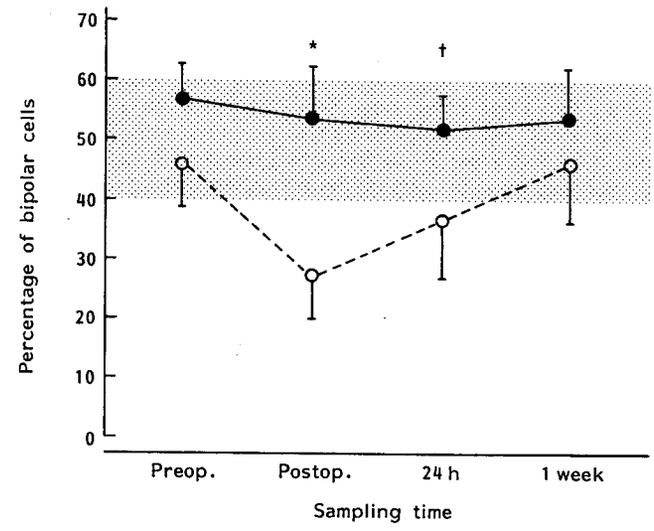

Figure 2 *Changes in bipolar shape formation in patients undergoing major vascular surgery with (●) or without (○) aprotinin. Values are median (95 per cent confidence interval); the normal range is indicated by the shaded area.* *P < 0·001; †P < 0·002 *(Mann–Whitney* U *test)*

Results

All patients completed the study. One individual in the aprotinin treatment group required a femoral embolectomy to retrieve a dislodged atheromatous plaque. Apart from this, there were no perioperative complications in either group, and grafts were functioning satisfactorily on follow-up at 3 months. Blood loss in the aprotinin-treated patients was significantly reduced as previously reported elsewhere[6].

White blood cell counts (*Figure 1*) rose immediately after surgery in both groups, although levels in aprotinin-treated patients remained significantly higher than in controls ($P < 0{\cdot}005$) at 24 h and 7 days after operation (*Figure 1c*). There was little difference in the total number of neutrophils, although the aprotinin group had significantly higher total ($P < 0{\cdot}02$) and percentage ($P < 0{\cdot}01$) neutrophil counts at the start of the study (*Figure 1b* and *a* respectively).

The effect of surgery on bipolar shape formation (BSF) is shown in *Figure 2*. Compared with the aprotinin group, BSF was significantly reduced in the control subjects immediately after surgery ($P < 0{\cdot}001$) and 24 h after operation ($P < 0{\cdot}002$). Furthermore, the BSF results at these times fell below the normal range. Aprotinin-treated patients, however, showed no significant fall in BSF and remained within normal limits throughout the operative period.

Compared with the aprotinin-treated patients, neutrophil migration towards FMLP was significantly reduced in the

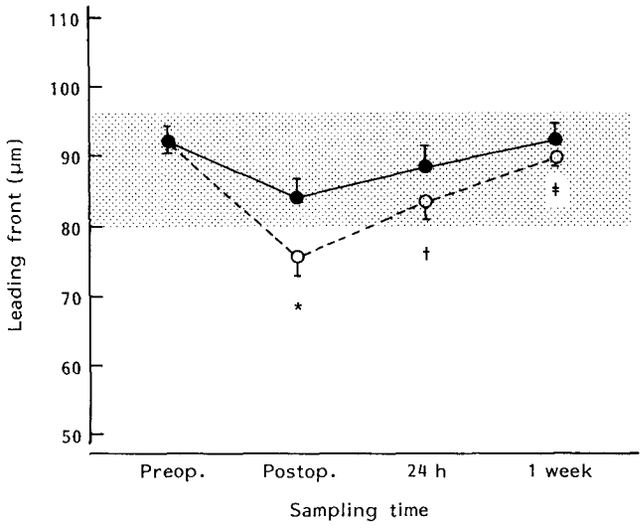

Figure 3 *Changes in neutrophil migration towards* N-*formylmethionyl-leucinylphenylalanine (10^{-9} mol/l) in patients undergoing major vascular surgery with (●) or without (○) aprotinin. Values are median (95 per cent confidence interval); the normal range is indicated by the shaded area.* *P < 0·001; †P < 0·002; ‡P < 0·05 *(Mann–Whitney* U *test)*

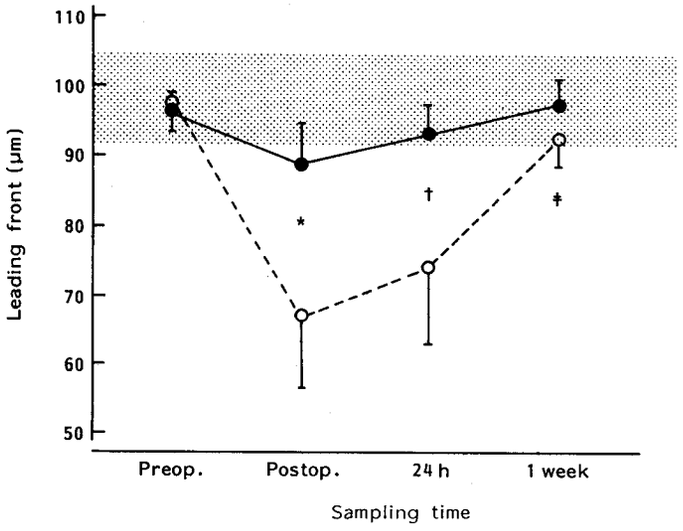

Figure 4 *Changes in neutrophil migration towards human AB serum in patients undergoing major vascular surgery with (●) or without (○) aprotinin. Values are median (95 per cent confidence interval); the normal range is indicated by the shaded area.* *P < 0·001; †P < 0·002; ‡P < 0·05 *(Mann–Whitney* U *test)*

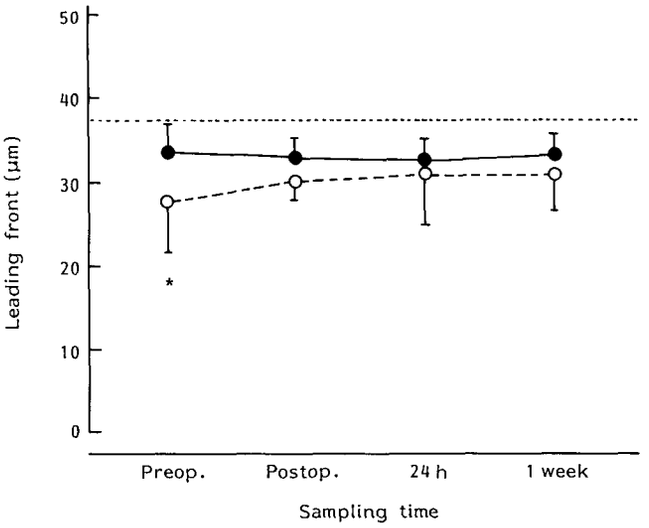

Figure 5 *Changes in non-directed (towards buffer) neutrophil migration in patients undergoing major vascular surgery with (●) or without (○) aprotinin. Values are median (95 per cent confidence interval); the upper normal limit is indicated by the dotted line.* *P < 0·05 *(Mann–Whitney* U *test)*

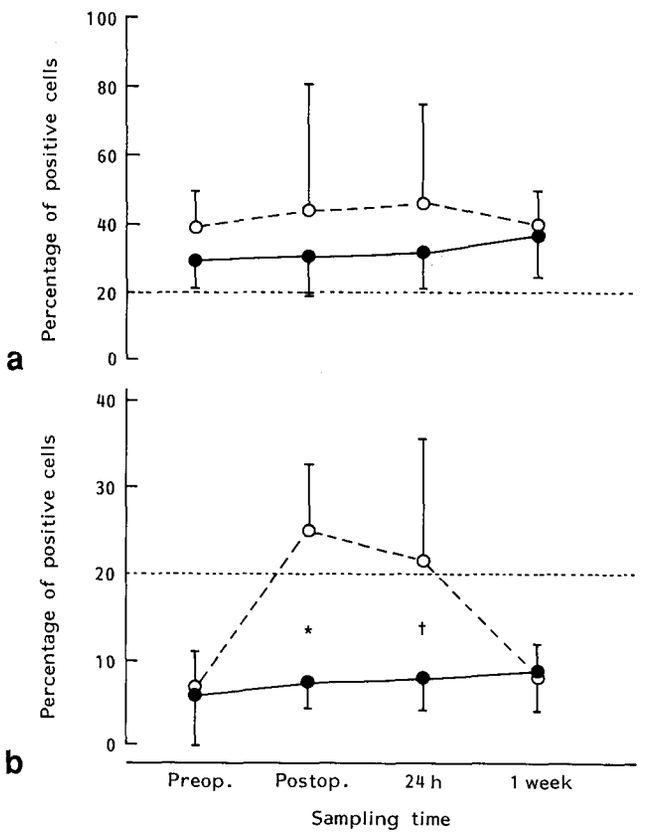

Figure 6 *Changes in* **a** *stimulated and* **b** *unstimulated nitroblue tetrazolium reduction in patients undergoing major vascular surgery with (●) or without (○) aprotinin. Values are median (95 per cent confidence interval); the* **a** *lower and* **b** *upper normal limits are indicated by the dotted lines.* *P < 0·001; †P < 0·02 *(Mann–Whitney* U *test)*

controls at all times after surgery, falling below the normal range for this test immediately after operation (*Figure 3*). Although the aprotinin group showed a broadly similar pattern, this was not as marked and migration always remained within the normal range. Changes in migration towards AB serum were more dramatic. The control subjects showed a marked reduction in migration, this becoming distinctly abnormal immediately after surgery, and remaining so for at least 24 h. Compared with that in the aprotinin group, this reduction was statistically significant at all times after surgery (*Figure 4*). Neutrophils from aprotinin-treated patients, however, showed only a slight decrease in migration over the operative period, falling just below normal limits immediately after the operation. There was no significant change in non-directed migration after surgery in either group, although the controls had a significantly lower ($P < 0.05$) baseline value. Nevertheless, all results were within the normal range for this technique (*Figure 5*).

Neutrophils from aprotinin-treated patients showed no change in resting (unstimulated) NBT reduction during the period of observation (*Figure 6b*). In contrast, cells from controls showed a marked rise in NBT reduction to levels that were above the normal range, and significantly greater than those in patients receiving aprotinin, immediately ($P < 0.001$) and 24 h ($P < 0.02$) after surgery. NBT reduction after stimulation with a bacterial extract was unchanged, and remained within normal limits, throughout the period of observation in both groups (*Figure 6a*).

Discussion

The results of neutrophil function tests in the control patients generally confirm the changes in perioperative neutrophil function reported by others. These include increases in total white cell count[21], reduction in intraneutrophilic granule proteins (indicating activation and release[22]), reduced killing capacity[23], diminished chemotactic ability[24–26], reduced superoxide production in response to FMLP or phorbol myristate acetate[27], and decrease in leukotriene B_4 production[22]. These defects may not all be directly attributable to surgery, however, because altered migrational ability, for example, may follow anaesthesia alone[24]. For this reason, baseline samples were taken after induction of anaesthesia in both groups. Previous studies were either of relatively brief duration[24] or carried out on mixed groups of patients, including some with malignancy, where disturbances in neutrophil function might be expected[27]. Duignan *et al.*[26] reported that chemotaxis was not significantly altered after surgery, although follow-up in this study was not until 5–8 days after operation. The present study suggests that neutrophil function has normalized by this time.

There are no previous reports of the effect of surgery on neutrophil BSF, although reduced oxidative responsiveness to FMLP, the stimulus used in this test, has been described[27]. The BSF test permits the rapid assessment of neutrophil responsiveness and is relatively simple compared with tests of migration. Because neutrophils change shape during locomotion, BSF may be functionally related to the locomotor capacity of these cells[17]. The fact that BSF remains unimpaired in aprotinin-treated patients, unlike the controls, suggests that it is a useful way of assessing locomotive ability after operation. Neutrophil migration was normal towards FMLP and AB serum in the aprotinin-treated group, but was significantly impaired in the control patients. This supports the results of the BSF assay and agrees with data reported elsewhere[24,28]. Taken together, the migration and BSF tests suggest that the ability to mobilize for movement and to migrate is better preserved in the aprotinin-treated patients than in the controls.

The results of the stimulated NBT reduction test in the aprotinin-treated patients were not significantly different from those in the controls, suggesting that the ability to respond fully to a bacterial stimulus was retained in the former group. These findings contrast with those of Utoh *et al.*[27], who found a diminished superoxide response to FMLP on the first day after operation, although the heterogeneous group of patients studied may have contributed to this difference. In the unstimulated NBT tests, however, significantly lower results in the aprotinin-treated patients suggest that, unlike the controls, their neutrophils were not already engaged in superoxide production. Taken together, the NBT test results suggest that the mechanisms for superoxide production remain intact in the aprotinin-treated patients and that the activation of this granulocyte function by surgery is prevented by aprotinin. The serial leucocyte and differential counts give little additional information except that the expected physiological neutrophil mobilization[29] is not impaired in the aprotinin-treated group.

Whether alterations in results of leucocyte function tests in patients with a normal immune capacity affect perioperative morbidity or mortality rates remains to be established. This subject has been debated since 1903 when Snel[30] and Rubin[31] showed that alterations in leucocyte function occurred under anaesthesia. More recently, Bruce and Wingard[32] found no correlation between neutrophil function and morbidity, and there does not appear to be any relation between preoperative neutrophil function and postoperative sepsis[26,28]. Neutrophils that are already stimulated and actively phagocytosing, or undergoing the respiratory burst, migrate relatively poorly (R. A. L., unpublished data) and the retention of the capacity to respond by both activation and migration seen in aprotinin-treated patients may be advantageous. There is no evidence that this would be detrimental to the patients' perioperative course.

There is no obvious explanation for the reduction in neutrophil activation during surgery in the aprotinin-treated patients. Damage to endothelial cells and release of platelet-derived products can activate neutrophils[33] and a platelet-protective effect may thus inhibit neutrophil activation. Activated components of the haemostatic and complement pathways may contribute. Kallikrein can activate neutrophils and release neutrophil elastase[34,35]. Aprotinin is a weak inhibitor of this enzyme[1], and plasma levels of elastase are lower in patients undergoing cardiopulmonary bypass treated with aprotinin[2]. This probably reflects lower levels of neutrophil activation rather than direct enzyme inhibition, as *in vitro* studies have shown that extracorporeal circulation, especially with bubble oxygenators, can greatly increase plasma elastase levels. As neutrophil elastase is thought to be an alternative pathway of fibrinolytic activation[36,37] and hyperfibrinolysis appears to play a role in the pathogenesis of surgical bleeding[38], such an effect may help to explain the haemostatic properties of aprotinin. The interaction between enzymes and other mediators of the inflammatory response is complex[39] and there may be many sites at which a proteolytic inhibitor could inhibit neutrophil activation.

These results show that aprotinin normalizes leucocyte function after major surgery. It seems unlikely, given the good response in the migration and NBT tests, that any potential leucocyte response is impaired. Many patients, especially those undergoing cardiac surgery, have been treated with high-dose aprotinin. There do not appear to be any harmful effects attributable to the effects of aprotinin on leucocyte function such as increased septic or thromboembolic complications. This study confirms, albeit in a limited number of patients, that aprotinin can be given as a haemostatic agent without inhibiting neutrophil function. The apparent preservation of neutrophil function may be of clinical benefit, although larger studies would be needed to confirm this suggestion.

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